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13. ABSTRACT ***(Maximum 200 Words)***

Accumulation of adipose tissue-derived fibroblasts around malignant epithelial cells provide critical structural and biochemical support for breast carcinomas. We present evidence that this process is mediated via complex interactions between malignant epithelial cells and undifferentiated adipose tissue fibroblasts. We demonstrated that the major mechanism for fibroblast accumulation in breast tumors is the inhibition of their differentiation to mature adipocytes via the action cytokines (IL-11, TNFα). Malignant epithelial cells inhibit adipocyte differentiation specifically via the suppression of expression of the essential adipogenic transcription factors C/EBPα and PPARγ. During this annual report period, we specifically determined the principal sources of IL-11 and TNFα and the distribution of expression of adipogenic transcription factors C/EBPα, C/EBPβ and C/EBPδ in sections of human breast tumors. We found that TNFα and IL-11 are primarily expressed in the malignant epithelial cell component of human breast tumors. Immunoreactive C/EBPα is not detectable in fibroblasts proximal to malignant cells, whereas in adipose tissue distant to the tumor, abundant amounts of C/EBPα were observed. In contrast, C/EBPβ and C/EBPδ are ubiquitously present in both intratumoral and distant adipose tissue fibroblasts. Thus, results obtained during this report period confirm that TNFα and IL-11secreted by malignant epithelial cells block adipocyte differentiation via specifically inhibiting the expression of the essential adipogenic factor C/EBPα. In contrast, C/EBPβ and C/EBPδ levels are upregulated by malignant cells. These two latter C/EBP isoforms are not essential nor sufficient for adipocyte differentiation.

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Serdar E. Bulun 12/23/99

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5. INTRODUCTION

This Annual Report was prepared as a requirement of the Idea Award entitled "Adipocyte Differentiation: Relationship to Breast Cancer" funded by the US Army Medical Research and Materiel Command Breast Cancer Research Program (DAMD17-97-1-7025). This report covers research for the period from 10/1/98 until 9/30/99. We should point out that, as of 2/22/99 until present, funding from this grant was interrupted due to the relocation of the PI from the UT Southwestern Medical Center to the University of Illinois at Chicago (UIC). Fortunately, the transfer procedures are almost complete, and the funding should start at UIC as of January 2000. Therefore, this report covers the actual research performed from 10/1/98 until 2/21/99. We were asked to revise the 10/1/97 – 9/30/98 report, since we did not realize report formats were changed. In the revised report submitted by 5/1/99, we included date generated not only during the 10/1/97 – 9/30/99 period but also during the following 5 months (10/1/98 – 2/21/99) at UT Southwestern Medical Center before the PI moved to UIC. Therefore, there will be a scientific overlap between this report covering 10/1/98 – 2/21/99 and the revised version of the previous annual report. For the present report, Judy Pawlus advised me to summarize the findings generated from 10/1/98 until 2/21/99 and refer the reviewers to the appendix, which represents a manuscript submitted recently to Cancer Research. We will initially provide an overview of this research work. Then, details will be provided in **6. BODY**. The long term objective of this project is to characterize the cellular and molecular mechanisms responsible for intra- and peritumoral accumulation of stromal fibroblasts. To achieve this objective, the following specific aims were proposed. The first **specific aim 1 is *to determine whether secretory products of breast cancer cells prevent differentiation of adipose fibroblasts into mature adipocytes***. Major goals in this aim have been accomplished and detailed in the previous annual report. **Specific aim 2 is *to characterize the secretory products of malignant epithelial cells, which downregulate adipogenic transcription factors***. This aim has also been accomplished almost completely and results were detailed in the previous annual report. **Specific Aim 3 is *to determine whether adipogenic transcription factors regulate aromatase P450 expression in human adipose fibroblasts.*** Progress could not be made due to interruption of funding . Studies will resume once the funding is restored. **Specific aim 4 is *to determine the regional distribution of C/EBPβ, PPARγ, C/EBPα and P450arom expression in the breast in relation to tumor location.*** During the current report period, we characterized the distribution of C/EBPα, C/EBPβ and C/EBPγ in adipose tissue fibroblasts proximal and distal to malignant epithelial cells in human breast specimens bearing tumors. During the current report period, we also determined that the malignant epithelial cells is the primary source of TNFα and IL-11 human breast tumor specimens.

6. BODY

Malignant breast epithelial cells induce a reaction in the adjacent adipose stroma characterized by accumulation of large numbers of fibroblasts, *i.e.*, desmoplastic reaction. This dense layer of peritumoral fibroblasts arises from the breast adipose tissue and provides structural and biochemical support for breast cancer. Here we seek to determine the epithelial-stromal interactions responsible for desmoplastic reaction using 3T3-L1 murine fibroblasts and human adipose fibroblasts, which differentiate to mature adipocytes as model systems. The following paragraph summarizes our findings from the previous annual report period:

After exposure to an activating cocktail of hormones for two days, control 3T3-L1 cells differentiated fully to mature adipocytes by days 6-8. Coculturing 3T3-L1 cells with T47D or MCF7 breast cancer cell lines inhibited this differentiation almost completely. Likewise, T47D-cell-conditioned medium gave rise to inhibition of the differentiation of 3T3-L1 cells. T47D-cell-conditioned medium also inhibited the differentiation of human breast adipose fibroblasts in primary culture, whereas control cells differentiated to mature adipocytes. This tumor effect was eliminated using neutralizing antibodies against TNFα or IL-11. TNFα mRNA was demonstrated by northern analysis in T47D cells treated with lipopolysaccharide or TNFα itself but not in 3T3-L1 cells indicating that T47D cells represent the major source of TNFα production in this co-culture system. Adipocyte differentiation is mediated by the expression of a cascade of adipogenic transcription factors including C/EBPδ or C/EBPβ, PPARγ and C/EBPα. We demonstrated by northern analysis that exposure of 3T3-L1 cells to T47D-cell-conditioned medium upregulated C/EBPδ or CEBPβ expression but suppressed or inhibited the expression of PPARγ and C/EPBα in 3T3-L1 cells treated with the cocktail. In these 3T3-L1 cells, inhibition of differentiation was also confirmed by markedly suppressed levels of aP2 mRNA, which is an adipocyte-specific gene. Treatment of 3T3-L1 cells with T47D cell-conditioned medium or TNFα changed neither the numbers of cells in G_O/G_1 or S phases nor the rate of [^{3}H]thymidine incorporation into these cells, thus, ruling out a proliferative effect of malignant cells on the surrounding fibroblasts. In summary desmoplastic reaction primarily occurs via the action of cytokines (TNFα and IL-11) secreted by the malignant epithelial cells to inhibit differentiation of adipose fibroblasts to mature adipocytes. This tumor-induced block in adipocyte differentiation is mediated by the inhibition of PPARγ and C/EBPα expression.

During the current report period, we employed immunohistochemistry to determine the distribution of C/EBPα, C/EBPβ and C/EBPγ in human mastectomy specimens. These results confirmed our findings and hypothesis summarized in the previous paragraph: We used 30 biopsies from 10 mastectomy specimens removed for breast cancer. We determined the expression of C/EBPα, C/EBβ and C/EPBδ in fibroblasts within the tumor sample (intratumoral), within adipose tissue biopsied 1 cm from the tumor (adjacent) and within adipose tissue biopsied 4 cm from

the tumor (distant). Fig 3 A-G in Appendix I illustrates fibroblasts and adipocytes with immunoreactive nuclei for these transcription factors. C/EBPα was not detectable in intratumoral fibroblasts, but it was readily detectable in fibroblasts and adipocytes in adjacent and distant fat tissue biopsies from 10 patients. No differences were observed in the distribution of expression of C/EBβ and C/EPBδ. An H-scoring system was used to determine the number of immunoreactive fibroblasts, as described in Materials and Methods (Fig 4 in Appendix I). This illustrated strikingly suppressed expression of C/EBPα in intratumoral fibroblasts. These results are in agreement with those of the northern analysis (Fig 2 in Appendix I).

Previous experiments have suggested that IL-11 and TNFα (Figure 1C in Appendix I) mediated the inhibition of adipocyte differentiation by cancer cells. Thus, during this report period, we determined the cellular origin of these cytokines in the breast cancer. First, we demonstrated TNFα transcripts in stimulated T47D breast cancer cells but not in 3T3-L1 cells employing northern blotting. (Fig 5 in Appendix I). A full-length murine TNFα cDNA was used to probe the membrane. Next, we determined the *in vivo* cellular distribution of immunoreactive IL-11 and TNFα in 15 mastectomy specimens. All malignant epithelial cells showed intense cytoplasmic staining for these two cytokines, whereas less than 25% of fibroblasts were immunoreactive with considerably less staining intensity (Fig 6A-C in Appendix I).

7. KEY RESEARCH ACCOMPLISHMENTS

- We report hereby portions of a complex epithelial-stromal interaction between malignant breast epithelial cells and surrounding adipose fibroblasts (preadipocytes) to develop a model whereby malignant cells maximize the number of surrounding undifferentiated adipose fibroblasts and stimulate estrogen production in these fibroblasts.

- Malignant breast epithelial cells inhibit adipocyte differentiation specifically via the suppression of the essential adipogenic transcription factor C/EBPα but not C/EBPβ or C/EBPδ, as demonstrated by the detection of these nuclear factors in sections of human breast tumors C/EBPβ and C/EBPδ are not essential or sufficient by themselves for adipocyte differentiation.

- Malignant epithelial cells are the major sites of expression of IL-11 and TNFα, which inhibit adipocyte differentiation.

8. REPORTABLE OUTCOMES

The following manuscripts bear the acknowledgement of this grant:

1. Meng L, J Zhou, H Sasano, T Suzuki, K Zeitoun, and **SE Bulun**: TNFα and IL-11 secreted by malignant breast epithelial cells inhibit adipocyte differentiation by selectively downregulating C/EBPα and PPARγ: mechanism of desmoplastic reaction. *Cancer Research.* Submitted.

2. **Bulun SE**, K Zeitoun, H Sasano, and E Simpson: Aromatase in aging women. Seminars in Reproductive Endocrinology. In press (2000).

3. Zeitoun K and **SE Bulun**: Aromatase: a key molecule in pathophysiology of endometriosis and a therapeutic target. *Fertility and Sterility*. 72:961-969 (1999).

9. CONCLUSIONS

Our results generated during this report period supported the central hypothesis in this grant application. Desmoplastic reaction, *i.e.*, intra- and peritumoral accumulation of fibroblasts provide structural and molecular support for breast tumors. This process of accumulation of fibroblasts around malignant epithelial cells in breast tumors is an active event mediated by malignant cells via endocrine manipulation of the surrounding stromal cells. We demonstrated *in vivo* that anti-adipogenic cytokines such as IL-11 and TNFα are of malignant epithelial cell origin. We also went back to human mastectomy specimens bearing tumors to demonstrate that expression of the essential adipogenic transcription factor C/EBPα is inhibited proximal to malignant cells, but not in cancer-free regions of the breast. These findings complement the mechanistic experiments using co-cultures, which also demonstrated that C/EBPα is a key molecule in the malignant cell-mediated inhibition of adipocyte differentiation in breast tumors. We refer the reviewers to Appendix I for further details. Since the undifferentiated adipose tissue fibroblast is a major site of aromatase expression giving rise to estrogen production in breast tumors, our future studies will concentrate on the effects of adipogenic transcription factors on aromatase promoter activity in these fibroblasts.

10. REFERENCES

Please refer to references provided in the attached manuscript (Appendix I).

11. APPENDIX

One appendix (appendix I) that represents the manuscript, which was submitted to Cancer Research (see Reportable Outcomes) is attached.

TNFα and IL-11 secreted by malignant breast epithelial cells inhibit adipocyte differentiation by selectively downregulating C/EBPα and PPARγ: mechanism of desmoplastic reaction

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The sources of funding for the study:
NCI Grant CA 67167 and USAMRMC Grant DAMD17-97-1-7025 (to S.E.B.) and an American Association of Obstetricians and Gynecologists Foundation Fellowship Award (to K.M.Z.).

Unpublished data: Zhou J, Zeitoun KM, Simpson ER, Bulun SE. C/EBPβ, C/EBPα and PPARγ stimulate P450arom promoter II activity in human and murine adipose fibroblasts: part of an epithelial-stromal interaction in breast cancer. Submitted.

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ABSTRACT

The dense layer of fibroblasts that accumulate around malignant breast epithelial cells (*i.e.*, desmoplastic reaction) arises from the breast adipose tissue and provides structural and biochemical support for breast cancer. We report herein a number of epithelial-stromal interactions responsible for desmoplastic reaction in breast cancer using cultured 3T3-L1 murine fibroblasts and human adipose fibroblasts, which can be activated with a cocktail of hormones to differentiate to mature adipocytes. Adipocyte differentiation was inhibited by co-culturing fibroblasts with various breast cancer cell lines (T47D, MCF7, SSC202, SSC78 and SSC30) completely or by breast cancer cell-conditioned media in a dose-dependent manner. This tumor effect was eliminated using neutralizing antibodies against TNFα or IL-11. Immunoreactive TNFα and IL-11 were readily detectable in malignant epithelial cells but not in the majority of the surrounding fibroblasts in histologic sections of breast tumors, and TNFα transcripts were detectable in T47D but not in 3T3-L1 cells in culture, indicating that the malignant epithelial cell is the major site of cytokine production. Adipocyte differentiation is mediated by the expression of a cascade of adipogenic transcription factors including C/EBPβ, C/EBPδ, PPARγ and C/EBPα. C/EBPα and PPARγ are essential for this process. We demonstrated by northern analysis that exposure of activated 3T3-L1 cells to T47D-cell-conditioned medium potentiated the induction of C/EBPβ and C/EBPδ transcript levels and strikingly decreased the levels of PPARγ and C/EBPα transcripts. In these 3T3-L1 cells, inhibition of differentiation was also confirmed by markedly suppressed levels of aP2 mRNA,

which is an adipocyte-specific gene. In 10 human mastectomy specimens bearing malignant breast tumors immunoreactive C/EBPα was readily detectable in adipose fibroblasts distant to the tumor but not in intratumoral fibroblasts, whereas immunoreactive C/EBPβ and C/EBPδ were present in fibroblasts from both sites. Treatment of 3T3-L1 cells with T47D cell-conditioned medium or TNFα changed neither the numbers of cells in G_0/G_1, S and G_2 phases nor the rate of [^{3}H]thymidine incorporation, thus, ruling out a proliferative effect of malignant cells on the surrounding fibroblasts. In summary, desmoplastic reaction primarily occurs via the action of cytokines (TNFα and IL-11) secreted by the malignant epithelial cells to inhibit differentiation of adipose fibroblasts to mature adipocytes. This tumor-induced block in adipocyte differentiation is mediated by the selective inhibition of expression of the essential adipogenic transcription factors, namely, PPARγ and C/EBPα.

INTRODUCTION

The group of infiltrating duct carcinomas constitutes 70 percent of all malignant mammary tumors (1). The neoplastic glandular formations (epithelial cells) are disseminated in a stroma that is comprised of mature adipocytes and fibroblasts. This tumor type, referred to commonly as "scirrhous" because of its extremely hard consistency, contains large numbers of fibroblasts dispersed between malignant epithelial cells, as well as within the immediate periphery of the tumor (desmoplastic reaction). The relationship between adipose stroma and breast cancer is unique in the sense that stromal fibroblasts seem to provide the structural support for cancer growth, whereas malignant cells greatly influence the composition of the adjacent tissue. Evidence from several laboratories indicates that this epithelial-stromal interaction also involves paracrine mechanisms that promote the development and growth of breast carcinomas (2-4). These morphologically identified intra-and peritumoral fibroblasts originate from adipose tissue and most likely represent potential pre-adipocytes, since the fibroblasts isolated from adipose tissue are capable of differentiating to mature adipocytes under defined culture conditions (5,6). We hypothesized that malignant epithelial cells of breast tumors secrete growth factors and cytokines to prevent the differentiation of fibroblasts to mature adipocytes in the adjacent adipose tissue. The following body of preliminary data supported this hypothesis. Firstly, tumors were found in breast quadrants with the highest aromatase P450 (P450arom) transcript levels and the highest fibroblast-to-adipocyte ratios (7). This parallelism between the distribution of fibroblasts and aromatase expression is not surprising, since aromatase is a marker for undifferentiated adipose fibroblastic cells (8,9). Secondly, in the cancer-free human

breast, the highest fibroblast-to-adipocyte ratios and P450arom transcript levels were found in the lateral and upper regions (10). This distribution roughly correlates with the most common sites where infiltrating duct carcinomas develop. Thirdly, breast tumor-conditioned media was found to induce aromatase expression in adipose fibroblasts (11). This tumor-induced effect can be inhibited and titrated by addition of an anti-IL-11 antibody (our unpublished observations). When aromatase expression is viewed as a fibroblast marker, these results suggested that malignant epithelial cells secrete factors such as IL-11 to prevent differentiation of fibroblasts. Additionally, estradiol pretreatment of cancer cells potentiated aromatase induction in a dose-dependent fashion, which is suggestive of a paracrine loop (11). Moreover, we demonstrated that certain adipogenesis inhibitors such as IL-11 are secreted by the T47D breast cancer cell line (12). Again, estradiol stimulated IL-11 expression in T47D cells in a dose-dependent fashion (12). Finally, IL-11 (13) and TNFα (14) stimulation of the fibroblast marker aromatase in adipose fibroblasts can be interpreted as inhibition of adipocyte differentiation, since aromatase expression in adipose tissue primarily resides in fibroblasts but not in mature adipocytes (8,9).

There may be multiple potential mechanisms responsible for accumulation of adipose fibroblasts within the tumor and in adjacent stroma. It is possible that fibroblasts proliferate in response to tumor-derived growth factors. Although treatment of murine 3T3-L1 fibroblasts in culture with serum, insulin, IGF-II and EGF initially causes proliferation; cell replication rapidly stops under these conditions, and fibroblasts eventually differentiate into mature adipocytes (15-18). Thus, it follows that there has to be other effective mechanisms for peritumoral fibroblast accumulation. Inhibition of

differentiation or dedifferentiation of preexisting adipocytes may provide these critical mechanisms responsible for extremely high fibroblast-to-adipocyte ratios in the stroma surrounding cancer cells. This process may be under the control of cytokines secreted by the malignant epithelial cells.

The cellular and molecular mechanisms responsible for the differentiation of stromal fibroblasts into mature adipocytes have been well-characterized. (19-24). During mammalian development, embryonic mesoderm gives rise to several highly specialized cell types, including adipocytes. Differentiation of adipocytes from multi-potential fibroblastic precursors is primarily controlled level by two tissue-specific transcription factors: the C/EBPα and PPARγ. During the process of differentiation, C/EBPβ (and possibly C/EBPδ) are initially expressed and convert multipotential mesenchymal precursor cells into pre-adipocytes (19). These "determined" pre-adipocytes are able to respond subsequently to potent adipogenic inducers such as PPARγ. The PPAR isoform, PPARγ (21) is a member of the ligand-activated transcription factor family that heterodimerizes with RXRα and binds to the promoters of adipocyte-specific genes. A third adipocyte-enriched transcription factor, C/EBPα, has been shown to promote the terminal adipocyte differentiation (20). When expressed together, PPARγ and C/EBPα act synergistically to powerfully promote adipocyte differentiation in fibroblastic cells regardless of tissue of origin (22,24). For practical purposes, we will refer to undifferentiated mesenchymal precursors and determined pre-adipocytes in human adipose tissue as adipose fibroblasts, since both cell types appear as fibroblasts morphologically. Fibroblasts isolated from adipose tissue differentiate into adipocytes when cultured in a defined medium (5,6). On the other hand, certain substances such as

TNFα are not only capable of inhibiting adipocyte differentiation, but also of reversing it by suppressing the expression of PPARγ (25, 26). Most of the work in this field has been performed using rodent fibroblasts and has been related to obesity and diabetes. Possible roles of malignant epithelial cells in paracrine regulation of these transcription factors and on adipocyte differentiation have not been studied to date. We report herein a number of epithelial-stromal interactions in the breast cancer, which represent the cellular and molecular mechanisms responsible for the development and maintenance of desmoplastic reaction.

MATERIALS AND METHODS

Tissue acquisition

Breast adipose tissue was obtained from 5 patients undergoing reduction mammoplasty. These tissues were immediately processed for primary cultures of adipose fibroblasts. Breast cancer and surrounding adipose tissue samples were obtained from 25 mastectomy specimens for immunohistological detection of C/EBPs (α, β, δ), IL-11 and TNFα. These studies were conducted following protocols approved by the Institutional Review Boards of the UT Southwestern Medical Center at Dallas, University of Illinois at Chicago and Tohoku University School of Medicine in Sendai, Japan

Detection of transcripts of adipocyte-specific genes and cytokines by northern blotting

Total RNA was isolated from fibroblasts/adipocytes in culture, electrophoretically fractionated (10μg) and transferred to a charged membrane. Duplicate measurements of optical density (OD at 260 μm) were performed to equalize loading, which was confirmed by visual inspection of 18S and 28S RNA stained with ethidium bromide. Northern blots were hybridized with cDNA probes labeled by random priming using [^{32}P]dCTP. Complementary DNA templates for murine adipocyte P2 (aP2), C/EBPδ, C/EBPβ, PPARγ, C/EBPα and TNFα were kindly provided by Drs Steve McKnight, Gokhan Hotamisligil, Carole Mendelson and Bruce Beutler.

Cell cultures and differentiation of fibroblasts to adipocytes

We routinely perform primary cultures of human adipose fibroblasts as previously described (8). Differentiation of human adipose fibroblasts to mature adipocytes was performed following a modified protocol originally outlined by Hauner and coworkers (5). Breast adipose tissue obtained from women at the time of reduction mammoplasty was processed through the mincing, washing, digestion with collagenase and centrifugation steps. The floating mature adipocytes were aspirated, and the sedimented fibroblast fraction was resuspended in DMEM with 10% fetal calf serum (FCS) as previously described (8). Nucleus-containing cells were inoculated at a density of 50,000/cm^2 into 35 mm dishes. Cultures were grown for a 24 hour period in DMEM with 10% FCS. Cells were then placed in a chemically defined phenol red-free and serum-free medium consisting of DMEM/Ham's F-12 medium (1:1, v/v), 15 mM NaHCO3, 15 mM hepes, 33 μM biotin, 17 μM pantothenate, 0.67 μM human insulin, 0.2 mM triiodothyronine, 0.5 mM dexamethasone (DEX) and antibiotics for 21 days. Within 15 to 21 days, cells achieved maximum differentiation. Cells were regarded as differentiated by morphological criteria when, after acquiring a round shape, their cytoplasm was completely filled with multiple lipid droplets as assessed by Oil Red O staining. The proportion of differentiated cells is estimated by direct counting under the microscope of total and differentiated cells, using a micrometer.

The 3T3-L1 fibroblasts were grown in DMEM with 10% FCS. T47D cells were initially grown in RPMI 1640 with 10% FCS containing 0.02 mM hepes, whereas MCF7 cells were grown in MEM with 10% FCS until confluent. SSC202, SSC78 and SSC30 breast cancer cells (kindly provided by Dr. Adi Gazdar) were grown to confluence in

DMEM with 10% FCS. All cells were incubated at 37°C, in 5% CO_2. To induce the adipogenic differentiation of 3T3-L1 fibroblasts within 2 days of reaching confluence, these cells were treated with DEX (0.25 μm), 1-methyl-3-isobutylxanthine (MIX, 0.5 mM) and insulin (1 μg/ml) for 2 days and then maintained in DMEM with 10% FCS for 6 additional days. Cells containing multiple fat droplets were scored as differentiated by phase contrast microscope after staining with Oil Red O. All culture media were phenol red-free.

Co-cultures of 3T3-L1 fibroblasts with breast cancer and human adipose fibroblasts with breast cancer cells were performed using 35 mm dishes and 25 mm permeable inserts. 3T3-L1 cells or adipose fibroblasts were plated in the bottom 35 mm dishes. Breast cancer cells were seeded on permeable membranes (pore size: 0.45 μm), which were inserted into these dishes. Both cells were exposed to the same media from the time they reached confluence. 3T3-L1 were cultured in DMEM with 10% FCS, after they reach confluence for two days. The medium was then changed to the appropriate differentiation medium for 48 h. Then the medium was switched back to DMEM plus 10% FCS for 6 days. In the case of human adipose fibroblasts, co-cultures were maintained for 15-20 days in the differentiating medium, since these cells required prolonged exposure to this medium in contrast to 3T3-L1 murine fibroblasts.

At the end of coculture experiments, human adipose fibroblasts or 3T3-L1 cells in the bottom plate were evaluated for differentiation to mature adipocytes and for proliferative indices (rate of [^{3}H]thymidine incorporation and flow cytometry) or were harvested for RNA isolation. Anti-human IL-11 (Ab-218-NA) and TNFα (Ab-210-NA) neutralizing antibodies were purchased from R&D Systems, Inc. (Minneapolis, MN).

Immunohistochemistry

Anti-human IL-1l, TNFα, C/EBPα, C/EBPβ and C/EBPδ antibodies were purchased from the R&D systems, Inc. (Minneapolis, MN). The immunohistochemical procedures were performed, as previously described, on 2.5-μm-thick sections mounted on poly-L-lysine-coated slides using the biotin-streptavidin amplified technique with a Histone immunostaining kit (Nichirei, Tokyo, Japan). Briefly, this staining procedure was performed as follows: 1) routine deparaffinization; 2) inactivation of endogenous peroxidase activity with 0.3% H_2O_2 in methyl alcohol for 20 min at 23°C; 2) blocking with 1% goat serum for 45 min at 23°C; 4) incubation with the primary antibody at 4°C for 18 h; 5) incubation with biotinylated goat anti-rabbit antibody for 30 min at 23°C; 6) incubation with peroxidase-conjugated streptavidin for 30 min at 23°C; 7) colorimetric reaction with a solution containing 0.05% Tris-HCl ([pH 7.6), 0.66 mol/L 3,3'-diaminobenzidine and 2 mol/L H_2O_2 and 8) counterstaining with 1% methyl green. H-scoring was used to quantify the number of immunoreactive cells in the following manner. Score 0: 0-5% cells positive; score 1: 6-25% cells positive; score 2: 26-50% cells positive; score 3: 51-75% cells positive; score 4: 76-100% cells positive. For each immunostain, the average score of 50 randomly chosen high power fields were calculated in each slide. Then, a mean ± standard error of the mean (SEM) for each group of patients was calculated from these values. Two investigators independently performed the H-scoring with an inter-observer variation of less than 5%.

RESULTS

Effects of tumor cells on differentiation of fibroblasts to adipocytes

Confluent 3T3-L1 cells differentiate to mature adipocytes, within 4 or 6 days after a 48-hour treatment with the cocktail including insulin, DEX and MIX. At this stage, the cells appear rounded and possess numerous large cytosolic lipid spheres as revealed by Oil Red O staining (Fig 1A). The effect of T47D breast cancer cell line on the differentiation in the 3T3-L1 cells was evaluated with co-cultures or T47D cell-conditioned medium. T47D breast cancer cells completely inhibited the differentiation of 3T3-L1 cells (Fig 1B). The inhibition of differentiation by the T47D cell-conditioned medium was demonstrated to be dose-dependent, when this medium was serially diluted with DMEM (control medium, data not shown). Upon addition of neutralizing antibodies to human IL-11 and TNFα, this effect was totally reversed. In other words, neutralizing both of these cytokines reversed the inhibitory effect of breast cancer cells on adipocyte differentiation (Fig 1C). This reversal was partial, when either antibody was used separately. (data not shown).

Co-cultures of T47D cancer cells inhibited the differentiation of human adipose fibroblasts completely. Other breast cancer cell lines (MCF7, SSC202, SSC78, SSC30) also inhibited the differentiation of both 3T3-L1 murine fibroblasts and human adipose fibroblasts. Moreover, cell lines that originated from other malignant tissues such as the liver cancer cell line HepG2 and the choricarcinoma cell line JEG3 also inhibited the differentiation of 3T3-L1 fibroblasts to adipocytes (data not shown).

Expression of adipogenic transcription factors and aP2 in 3T3-L1 cells cocultured with breast cancer cells

We determined the levels of transcripts of C/EBPα, C/EBβ, C/EPBδ, PPARγ and aP2 by northern analysis in confluent 3T3-L1 cells exposed to the adipocytic differentiation cocktail only (DMEM + COC), the cocktail plus the T47D cell-conditioned medium (CM + COC) or culture medium only (DMEM, used as a control). Fig 2 (A and B) depicts these results. Treatment with TCM strikingly decreased the transcript levels of C/EBPα and PPARγ in 3T3-L1 cells, whereas it significantly potentiated the effects of the differentiation cocktail to induce C/EBPβ and C/EBPδ during days 4 and 6 after the initiation of treatments. As expected, TCM also inhibited the expression of aP2, which is a marker for adipocyte differentiation (Fig 2B). These results were confirmed by the *in vivo* cellular distribution of immunoreactive C/EBPα, C/EBPβ and C/EBPδ in 30 biopsies from 10 mastectomy specimens removed for breast cancer. We determined the expression of C/EBPα, C/EBβ and C/EPBδ in fibroblasts within the tumor sample (intratumoral), within adipose tissue biopsied 1 cm from the tumor (adjacent) and within adipose tissue biopsied 4 cm from the tumor (distant). Fig 3 (A-G) illustrates fibroblasts and adipocytes with immunoreactive nuclei for these transcription factors. C/EBPα was not detectable in intratumoral fibroblasts, but it was readily detectable in fibroblasts and adipocytes in adjacent and distant fat tissue biopsies from 10 patients. No differences were observed in the distribution of expression of C/EBβ and C/EPBδ. An H-scoring system was used to determine the number of immunoreactive fibroblasts, as described in Materials and Methods (Fig 4). This illustrated strikingly suppressed expression of

C/EBPα in intratumoral fibroblasts. These results are in agreement with those of the northern analysis (Fig 2).

Cellular localization of the cytokines inhibitory for adipocyte differentiation in breast cancer

Previous experiments have suggested that IL-11 and TNFα (Figure 1C) mediated the inhibition of adipocyte differentiation by cancer cells. Thus, we determined the cellular origin of these cytokines in the breast cancer. First, we demonstrated TNFα transcripts in stimulated T47D breast cancer cells but not in 3T3-L1 cells employing northern blotting. (Fig 5). A full-length murine TNFα cDNA was used to probe the membrane. Next, we determined the *in vivo* cellular distribution of immunoreactive IL-11 and TNFα in 15 mastectomy specimens. All malignant epithelial cells showed intense cytoplasmic staining for these two cytokines, whereas less than 25% of fibroblasts were immunoreactive with considerably less staining intensity (Fig 6A-B).

Effects of T47D breast cancer cells on the proliferation of 3T3-L1 fibroblasts

We determined whether T47D cancer cells affect the proliferation indices of 3T3-L1 fibroblasts when co-cultured. Figure 7 demonstrates no differences in the DNA histograms of 3T3-L1 cells incubated in the absence or presence of T47D cells using flow cytometry. Neither did we see an effect of T47D breast cancer cells on the [^{3}H]thymidine incorporation into 3T3-L1 cells. Thus, we conclude that breast cancer cells induce accumulation of fibroblasts in the tumor tissue by the inhibition of differentiation of these cells to mature adipocytes but not by promotion of their proliferation.

DISCUSSION

Peri- and intratumoral fibroblasts provide structural support to tumor tissue, and secretory products of fibroblasts may promote tumor growth. We herein demonstrated that malignant breast epithelial cells actively participate in the process of accumulation of stromal fibroblasts in and around the tumor tissue (*i.e.* desmoplastic reaction). Secretory products of cancer cells prevent the differentiation of fibroblasts to adipocytes and, in fact, may even reverse adipocyte differentiation. We also demonstrated that tumor-derived cytokines act on adjacent adipose stroma by downregulating the expression of adipogenic factors C/EBPα and PPARγ. This study provides evidence that breast cancer cells (or their secretory products) do not induce proliferation of fibroblasts. Thus, inhibition of differentiation seems to be the major mechanism responsible for desmoplastic reaction.

Malignant epithelial cells inhibit adipocyte differentiation by suppressing two essential adipogenic factors, C/EBPα and PPARγ. Since this effect of breast tumors is mediated by TNFα and IL-11, this finding is not surprising. TNFα inhibits the expression of C/EBPα and PPARγ in 3T3-L1 cells (25,27). Ectopic expression C/EBPα is sufficient to induce differentiation of fibroblasts without the use of external inducers, whereas ectopic expression of PPARγ accomplishes this in the presence of its ligands (23,24,28,29). Moreover, a strikingly cooperative relationship between C/EBPα and PPARγ is observed, when both factors are expressed in the same cells (24). On the other hand, ectopic expression of C/EBPβ in fibroblasts causes commitment to the adipose lineage. Adipocyte differentiation, in this case, however, requires the expression of

PPARγ, which is induced by C/EBPβ (19). The role of C/EBPδ, which has a much lower adipogenic potential than C/EBPβ, remains uncertain. It appears that both C/EBPα and PPARγ are essential for full adipocyte differentiation, whereas C/EBPβ or δ cannot accomplish this in the absence of C/EBPα or PPARγ. Thus, it is not surprising that the net effect of downregulation of C/EBPα and PPARγ and upregulation of C/EBPβ and δ is the blockage of adipocyte differentiation in breast tumors.

In contrast to C/EBPα and PPARγ, the levels of C/EBPβ and C/EBPδ transcripts increased in adipose fibroblasts during days 4 and 6 upon co-culturing with breast cancer cells. A number of investigators reported that treatment with TNFα increased nuclear concentrations of C/EBPβ and C/EBPδ via posttranscriptional mechanisms and also enhanced binding of C/EBPβ to target promoters in 3T3-L1 fibroblasts, macrophages and hepatocytes (27,30-32). Additionally, one group of investigators observed that TNFα induced C/EBPβ transcript levels in macrophages (32). Our similar observations in 3T3-L1 fibroblasts may represent a long term effect of TNFα or other secretory products of cancer cells. This is clinically relevant, since we demonstrated that C/EBPβ strikingly induces the activity of P450arom promoter II, which is alternatively utilized in fibroblasts in breast carcinomas (33, 34). P450arom catalyzes the conversion of C_{19} steroids to estrogens in adipose fibroblasts.

We chose to demonstrate to effects of TNFα and IL-11, because both cytokines are extremely potent inhibitors of adipogenesis (26,35). In fact, IL-11 had been independently cloned as adipogenesis inhibitory factor (35). Besides IL-11, it is likely that other members of the IL-6 cytokine family may mediate this anti-adipogenic effect of malignant cells as a fail-safe mechanism. Since neutralizing the effects of both TNFα

and IL-11 in the co-culture system fully restored adipocyte differentiation, and abundant expression of both cytokines were demonstrated in malignant breast cells; the roles of other cytokines were not investigated in this study.

Besides breast tumors, desmoplastic reaction is also observed in other malignancies such as melanoma and colon cancer. By the same token, breast cancer cells were not specific for the inhibition of adipocyte differentiation. In our hands, liver cancer and choriocarcinoma cell lines also produced a similar effect. The uniqueness of breast cancer as a striking model for desmoplastic reaction arises from the vulnerability of adipose tissue to infiltration by malignant epithelial cells, which are dependent for growth on structural support and estrogen provided by these fibroblasts. It may be possible to demonstrate analogous epithelial-stromal interactions in other types of malignancies.

This report lays the groundwork for the mechanism of desmoplastic reaction as an epithelial-stromal interaction in the breast cancer. Further studies are required to identify other key molecules in the cancer-mediated inhibition of adipocyte differentiation. Two candidate substances are CHOP, a transcription factor that acts as a negative dominant regulator of adipocyte differentiation and Pref-1, which is a transmembrane protein with EFG-like motifs and another negative regulator of adipocyte differentiation (36,37). The determination of the roles of these candidate substances will further increase our understanding of the epithelial-stromal interactions in the breast cancer.

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FIGURE LEGENDS

Figure 1.

Panel A: 3T3-L1 cells, 8 days after reaching confluence. Confluent cells were treated with the differentiating cocktail (dexamethasone, insulin and MIX) for the first 2 days and then were maintained in DMEM for the following 6 days. Accumulation of lipid droplets represented by the red stain was detected in the cytoplasms of 80% of adipocytes (stain: Oil Red O).

Panel B: Coculturing of 3T3-L1 fibroblasts with breast cancer cells (in this instance, T47D cells) completely inhibited their differentiation to adipocytes evident by the lack of lipid droplets.

Panel C: This cancer-induced inhibition of differentiation was totally reversed by the addition of neutralizing antibodies to IL-11 and TNFα. This indicated that cancer cells inhibited adipocyte differentiation, at least in part, by the secretion of IL-11 and TNFα.

Figure 2. Northern blot showing the levels of transcripts of C/EBPα, C/EBPβ, C/EBPδ **(Panel A)**, PPARγ and aP2 **(Panel B)** in 10 μg of total RNA from 3T3-L1 cells incubated under conditions explained in the legend of Figure 1. The 28S RNA fraction was included to demonstrate the presence of comparable amounts of RNA in each lane. (DMEM: control medium; COC: differentiating cocktail; TCM: T47D cell-conditioned medium). T47D cells (TCM) inhibited expression of C/EBPα and PPARγ, and stimulated expression of C/EBPβ or C/EBPδ in 3T3-L1 cells after exposure to the

differentiating cocktail (COC). The expression of aP2, a marker of differentiated adipocytes, was suppressed by medium conditioned with T47D cells (TCM).

Figure 3. **Panel A:** Immunoreactive C/EBPα is not detected in the nuclei of fibroblasts within the breast tumor (intratumoral). [Brown stain indicates immunoreactive adipogenic transcription factors.] **Panel B:** C/EBPα (arrows) is readily detectable in the nuclei of fibroblasts/adipocytes distant to the tumor evident by the lack of malignant epithelial cells. **Panel C:** C/EBPβ is readily detectable in the nuclei of intratumoral fibroblasts (large arrows) and malignant epithelial cells (small arrows). **Panel D:** C/EBPβ (arrows) is readily detectable in the nuclei of fibroblasts/adipocytes distant to the tumor. **Panel E:** C/EBPδ is readily detectable in the nuclei of intratumoral fibroblasts (large arrows) and malignant cells (small arrows). **Panel F:** C/EBPδ (arrows) is readily detectable in the nuclei of fibroblasts/adipocytes distant to the tumor.

Figure 4. Graphic representation of the data in Figure 3. The number of cells with immunoreactive proteins were determined in the 3 biopsies (intratumoral, adjacent fat, distant fat) from each of the 10 mastectomy specimens. Expression of C/EBPα in intratumoral fibroblasts were found to be strikingly lower compared with fibroblasts in breast adipose tissue adjacent or distant to the tumor.

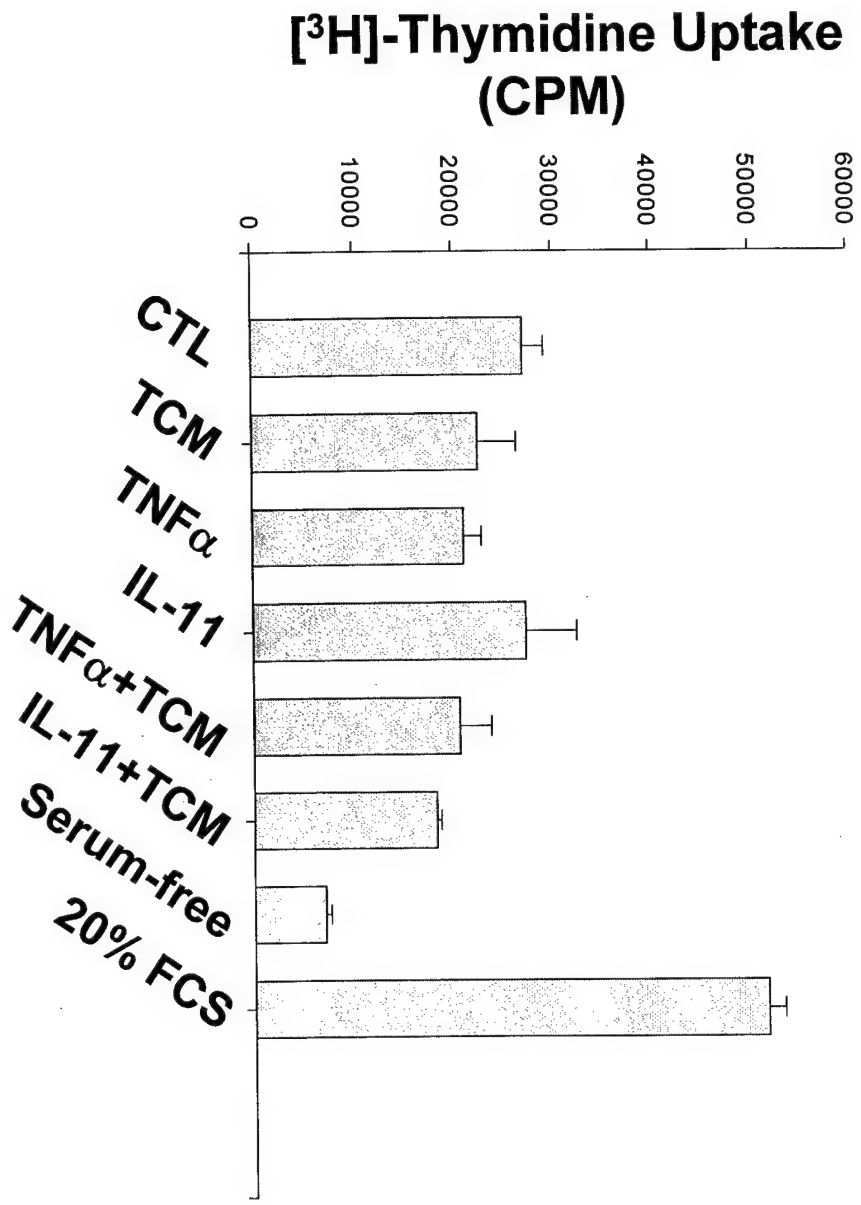
Figure 5. Northern blot analysis demonstrating TNFα transcripts in T47D breast cancer cells treated with lipopolysaccharide (LPS, 1μg/ml) and TNFα (10 mg/ml) for 3h. Under similar treatment conditions, transcripts were not detected in 3T3-L1 murine

fibroblasts. Murine RAW264.7 macrophage line was used as a positive control, whereas U937 human leukemia cell line was used as a negative control. Probe: full length murine TNFα cDNA. (The molecular weight of human TNFα transcripts in T47D cells was relatively lower, as expected.)

Figure 6. Panel A: Immunoreactive TNFα (brown stain) is readily detectable in malignant epithelial cells but not in fibroblasts. **Panel B:** Immunoreactive IL-11 (brown stain) is readily detectable in malignant epithelial cells but not in fibroblasts/adipocytes. **Panel C:** Graphic representation of the data. The number of cells with immunoreactive proteins were determined by H-scoring in the 2 cell types (malignant epithelial cells and fibroblasts) in each of the 15 tumor samples specimens.

Figure 7. No differences in the DNA histograms were detected in 3T3-L1 cells incubated without **(A)** or **(B)** with T47D breast cancer cells. The numbers of cells in G_0/G_1, S and G_2 were not significantly different.

Figure 8. Results of [^{3}H]thymidine uptake by 3T3-L1 cells incubated under various conditions represented as mean ± SEM (standard error of mean) of 9 replicates. Fetal calf serum (FCS, 20%) was used as a positive control, whereas serum-free medium was used as a baseline control. FCS (0.1%) was added to the rest of the treatments. The first column (CTL, control) represents 0.1% FCS only. CPM, counts per minute; TCM, tumor conditioned medium.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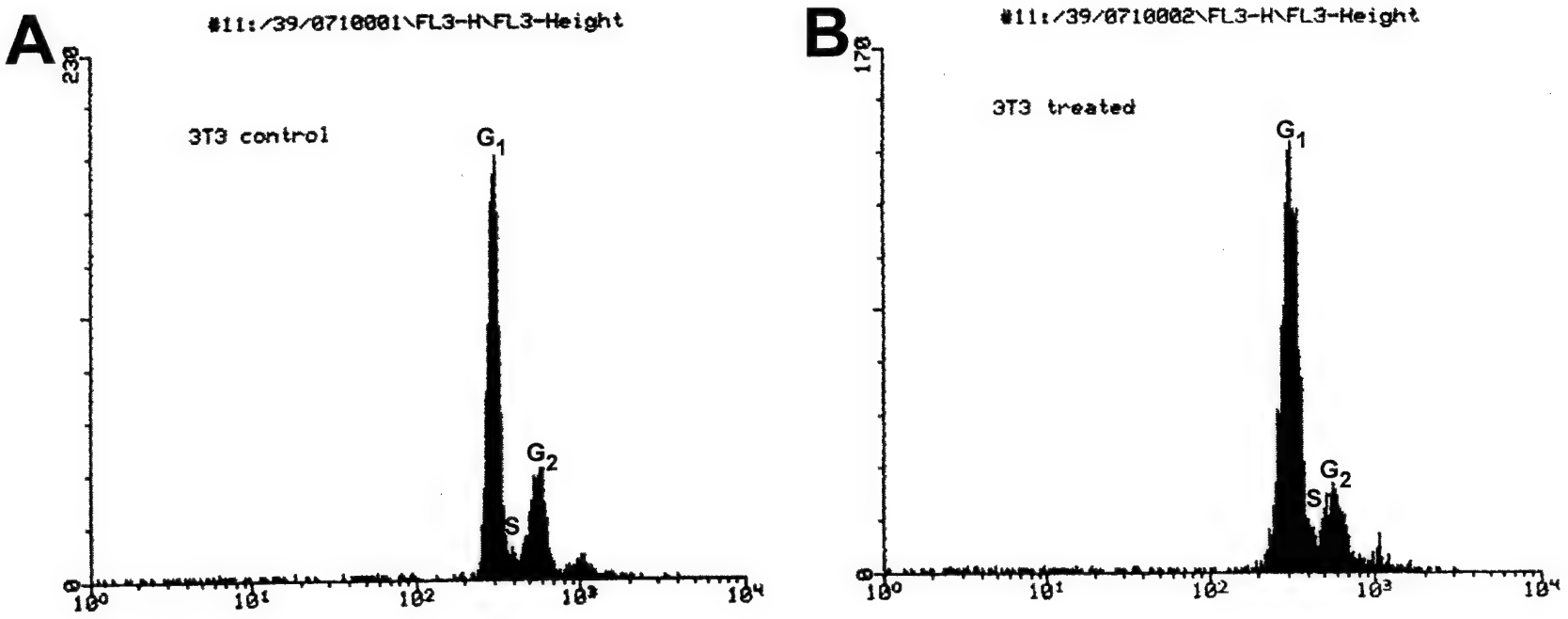
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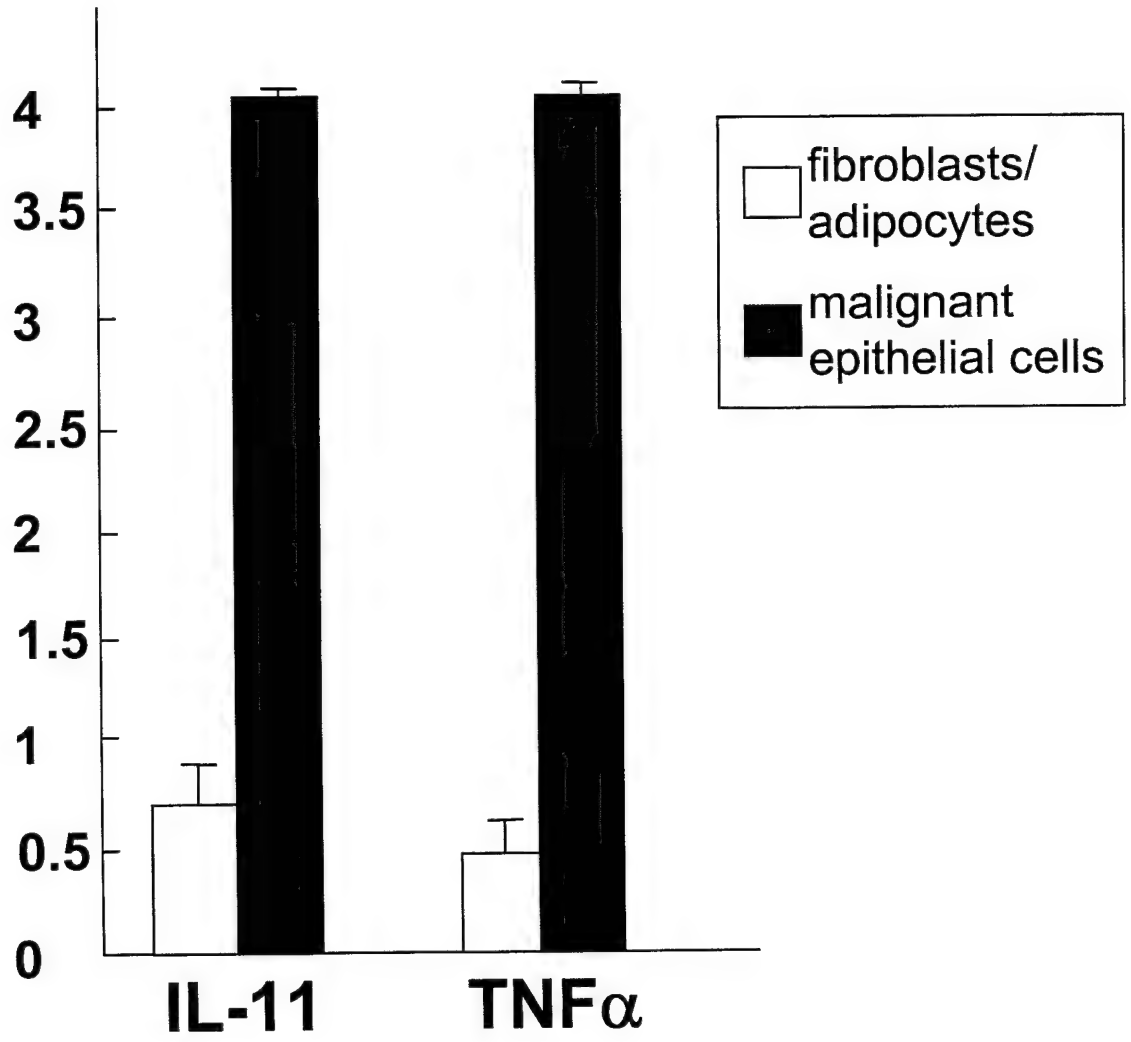
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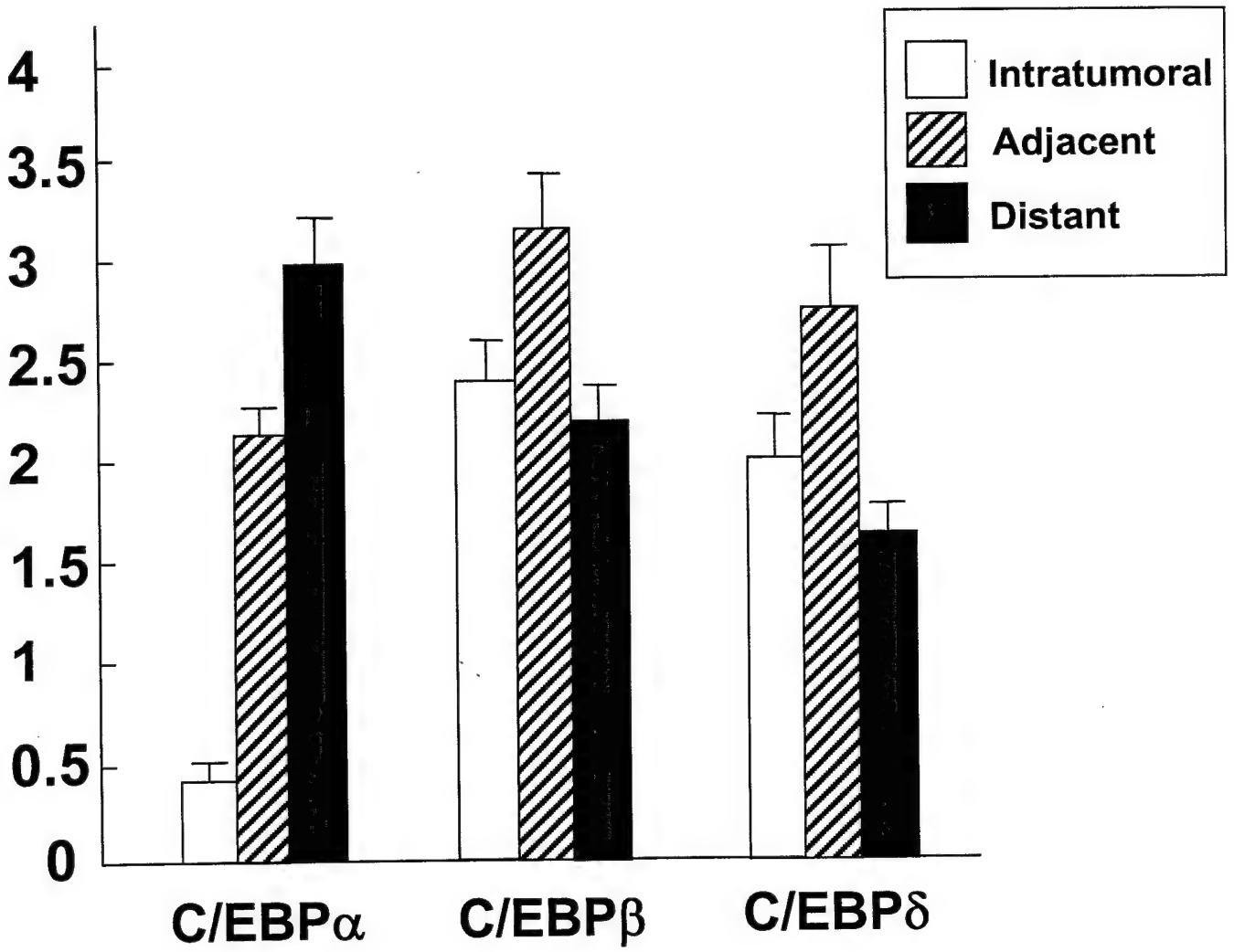
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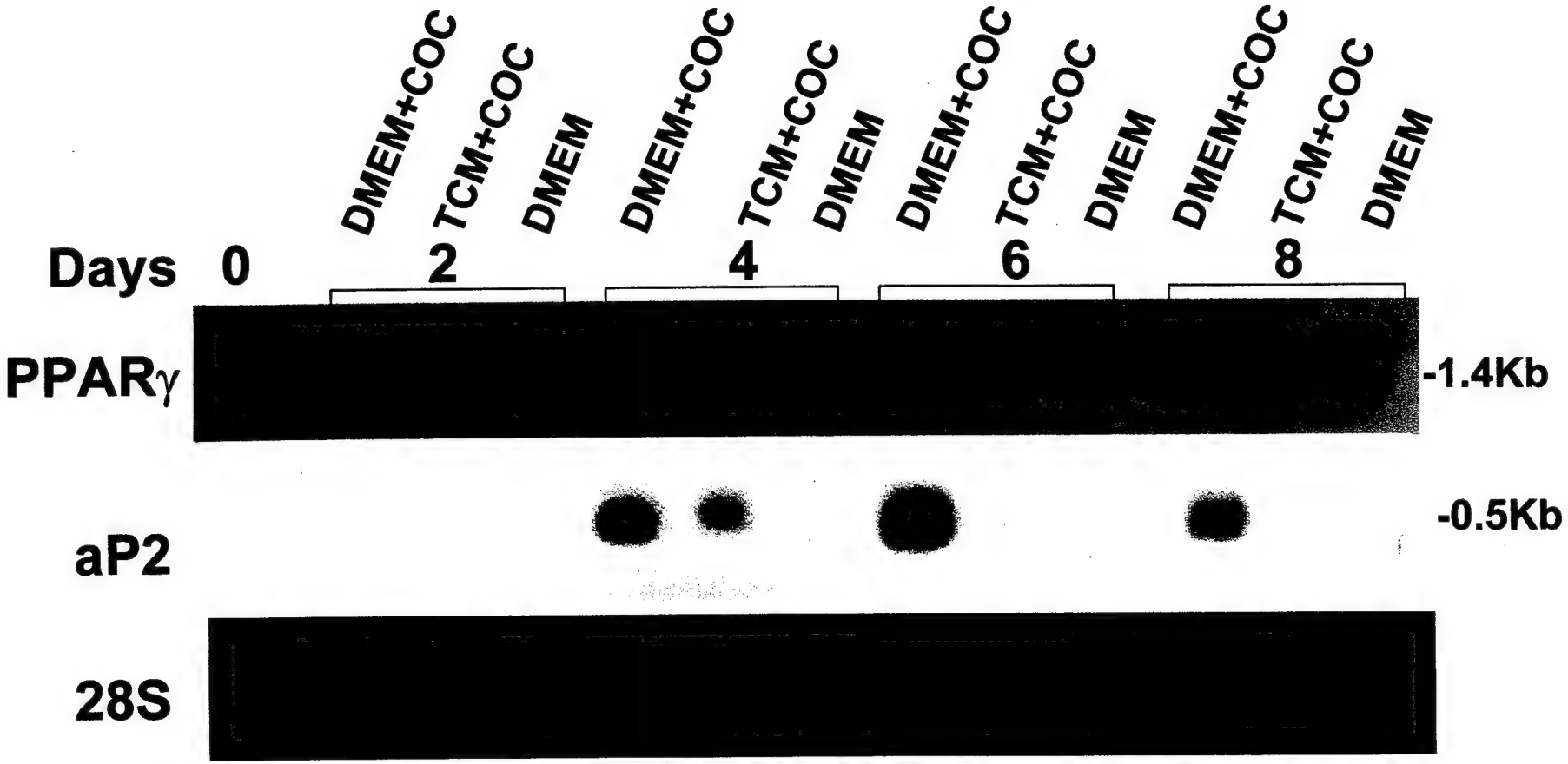
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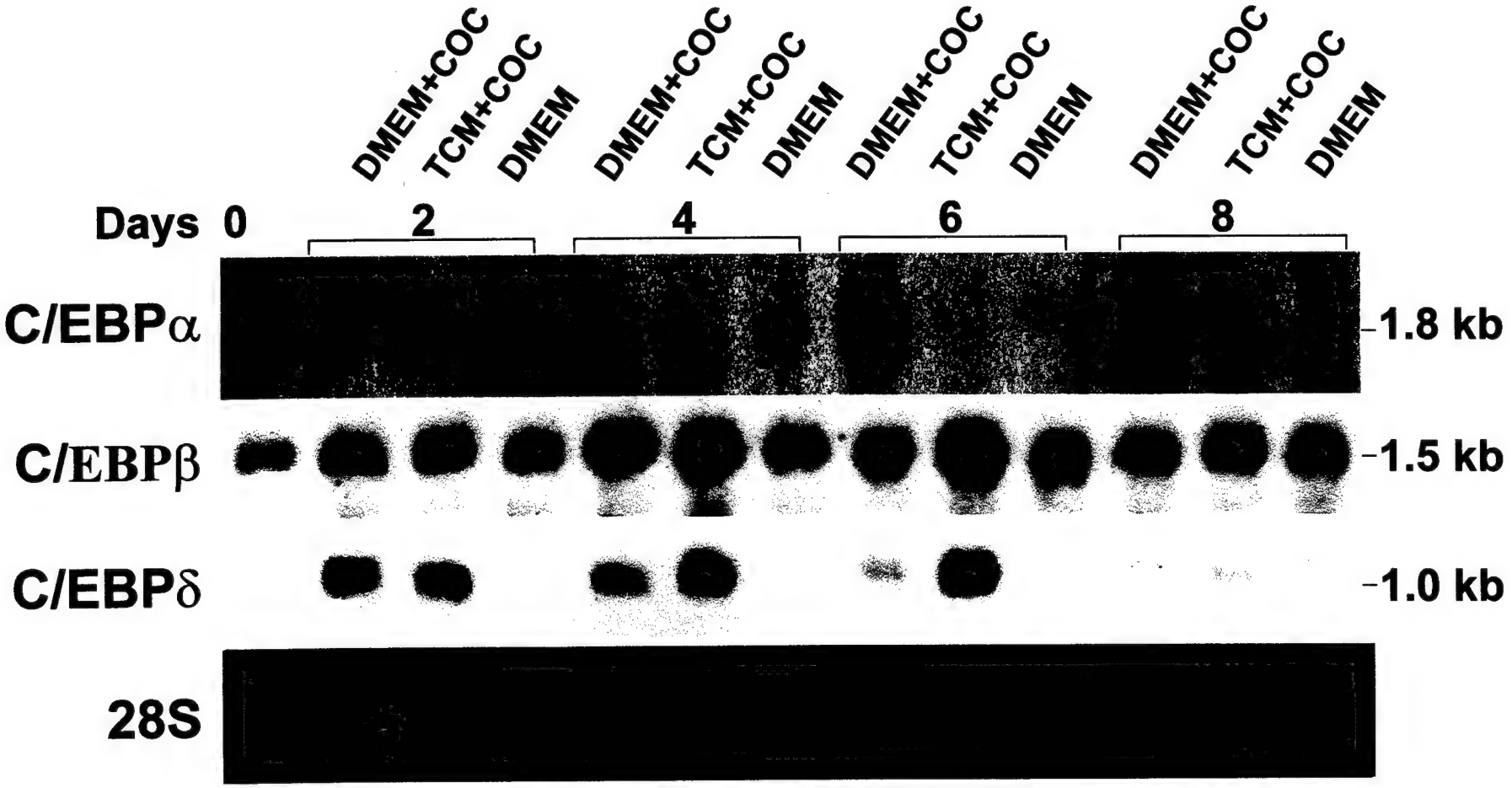
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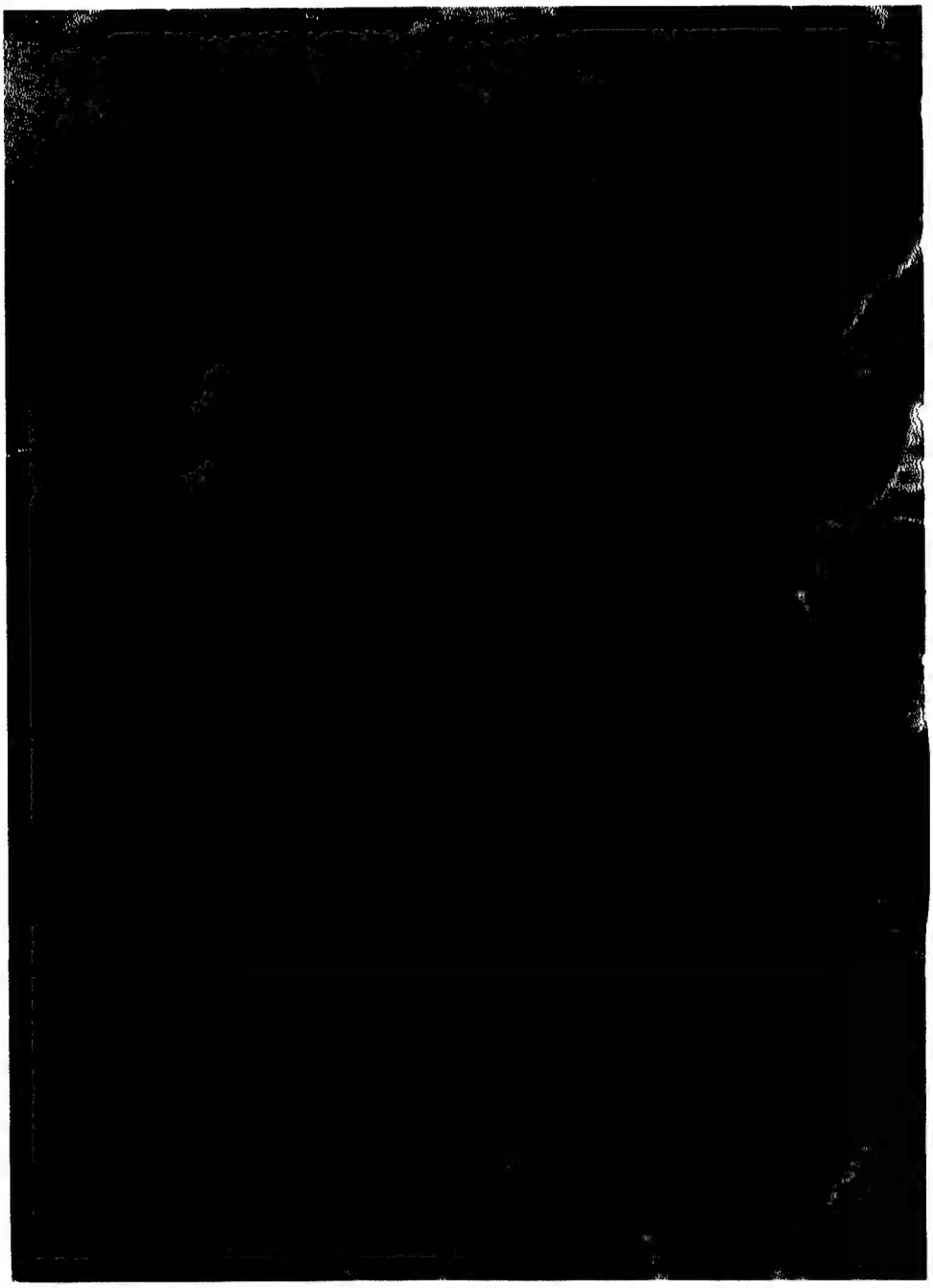

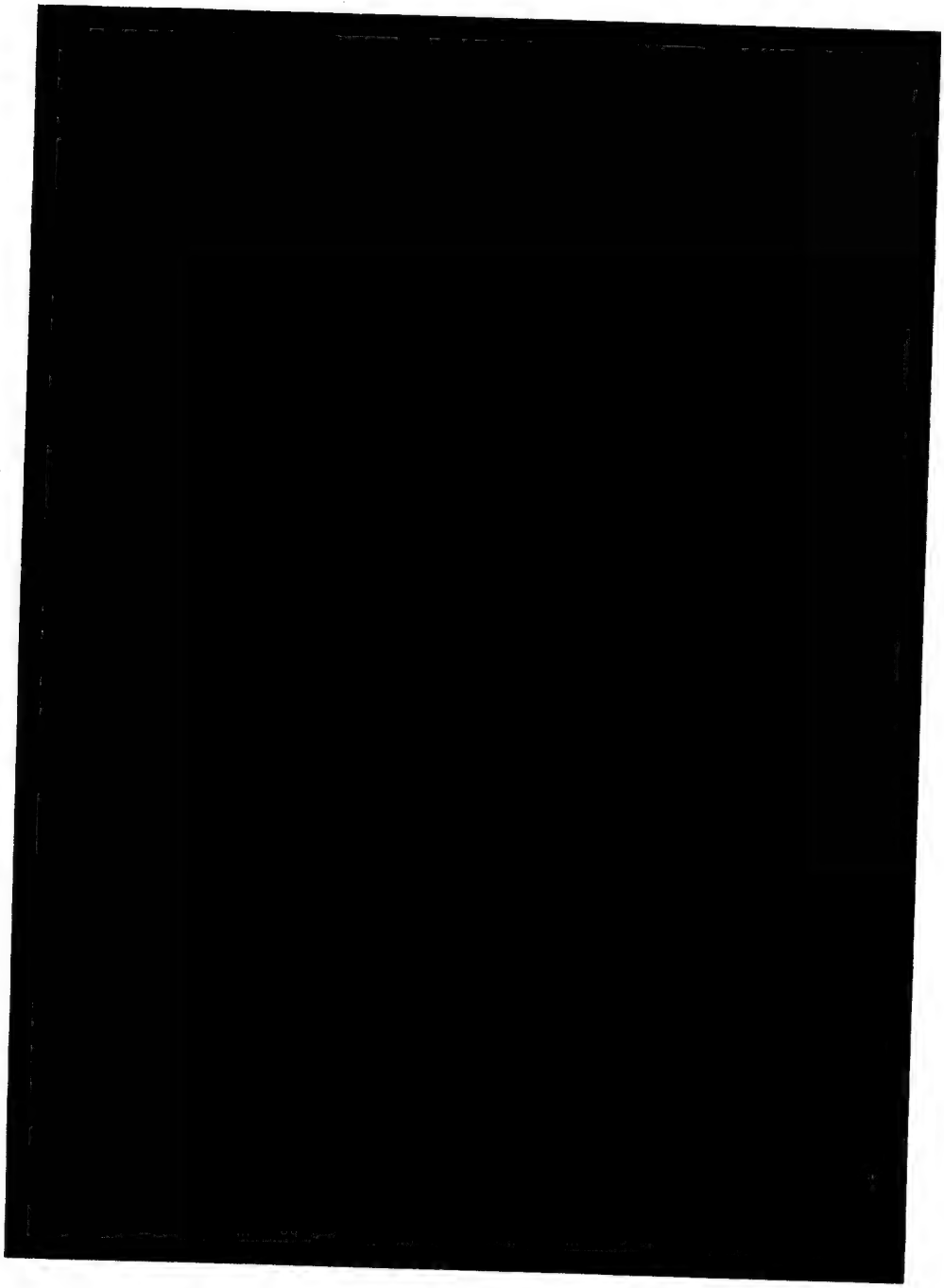

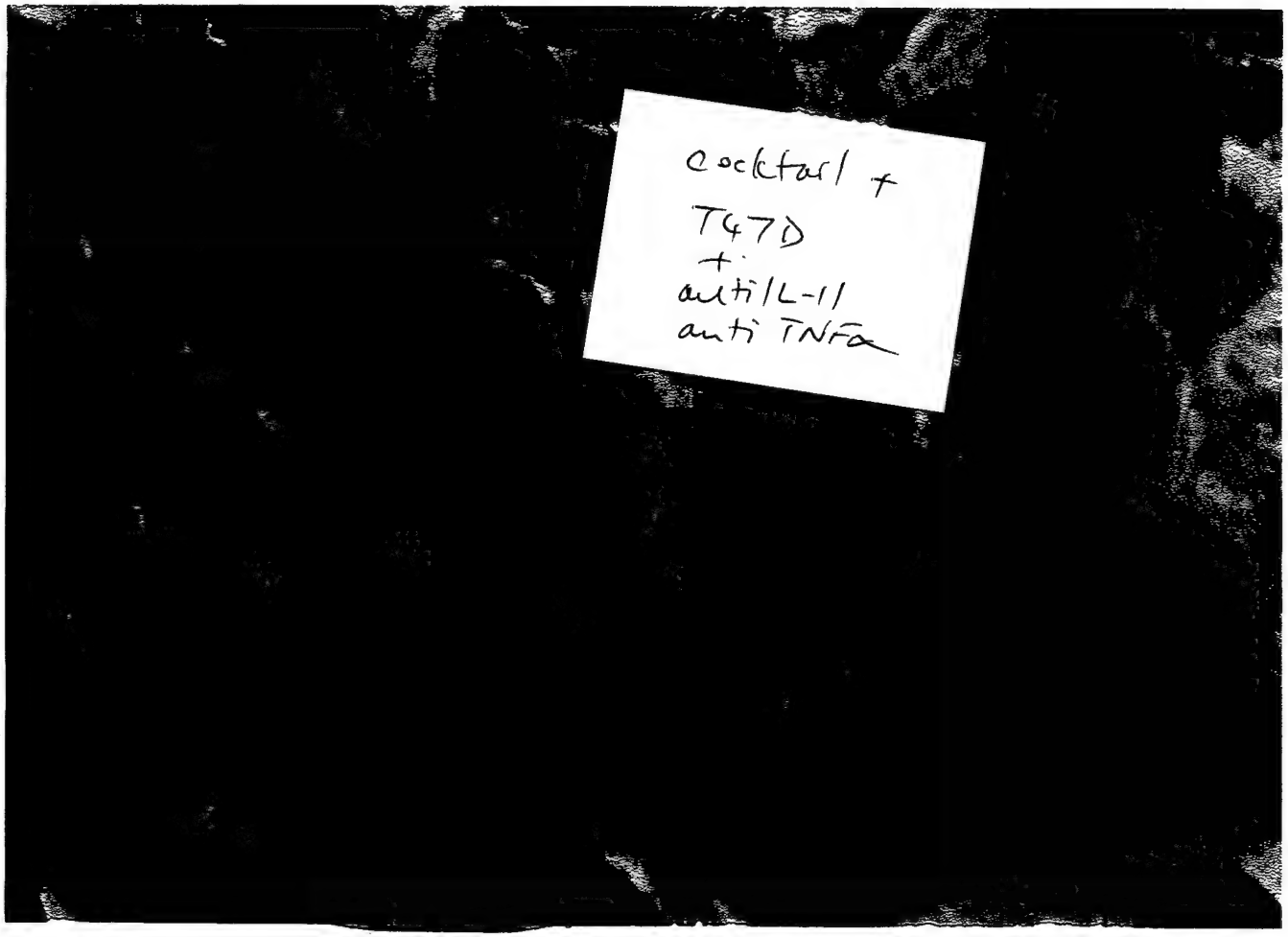
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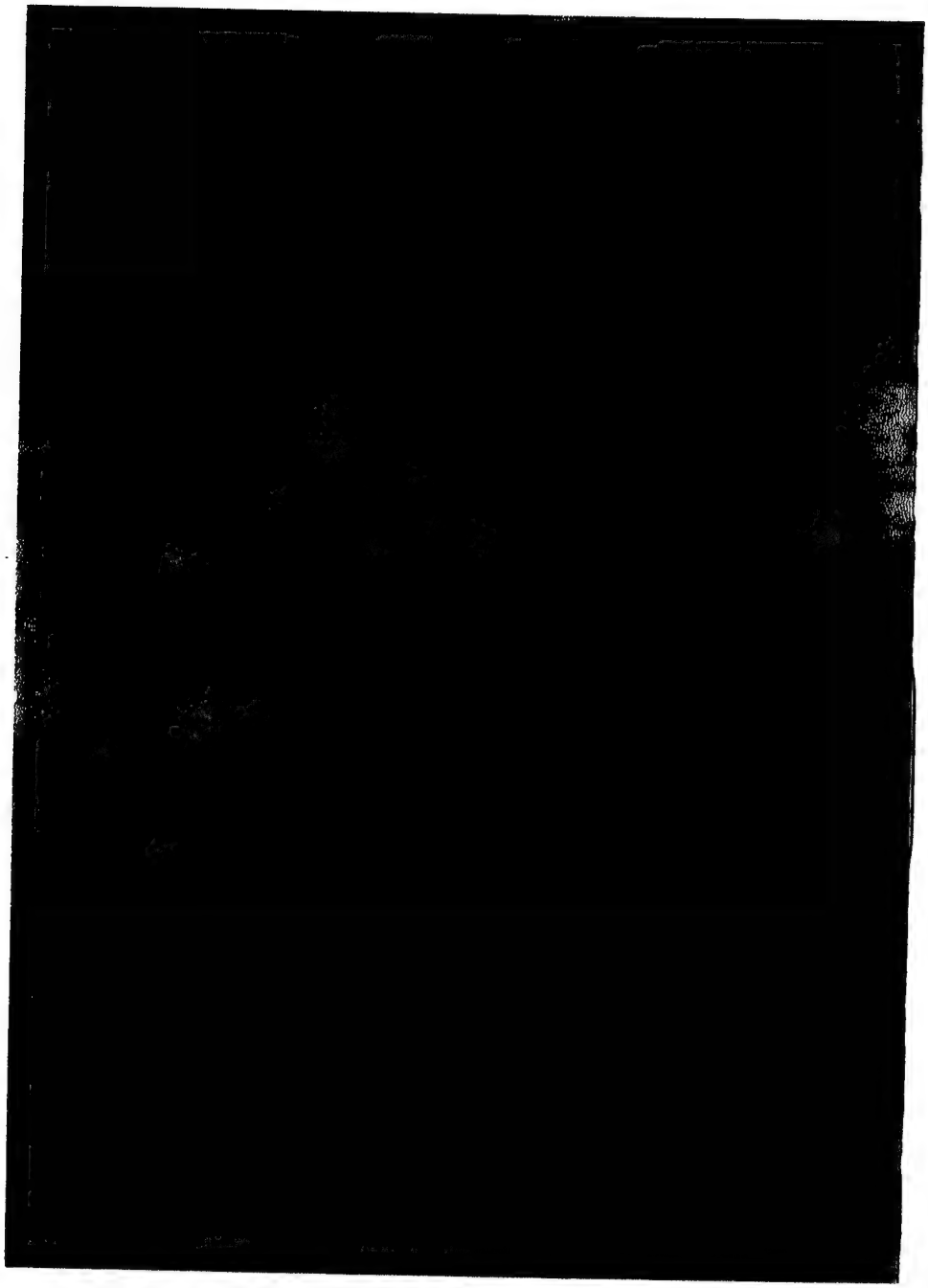

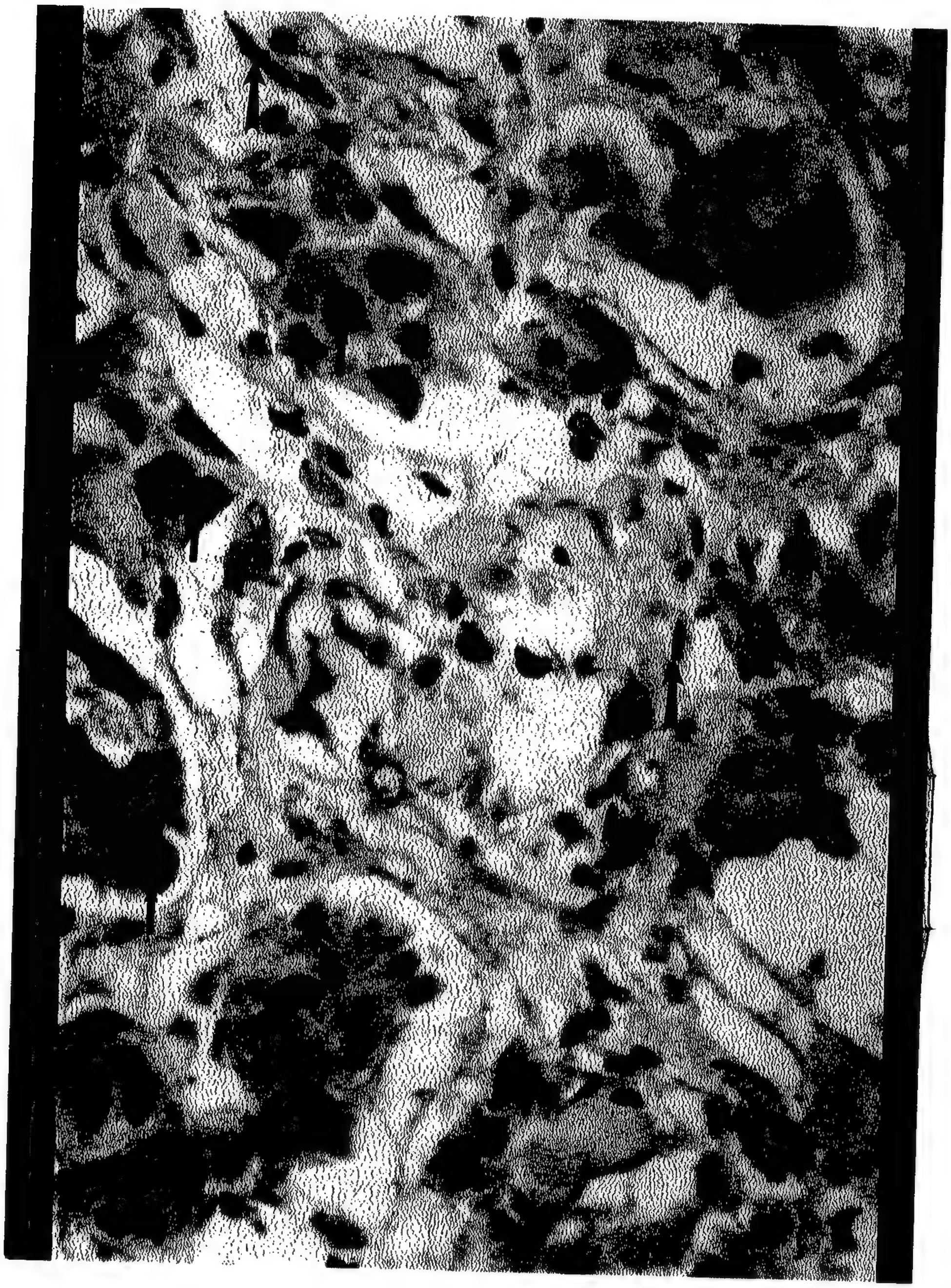

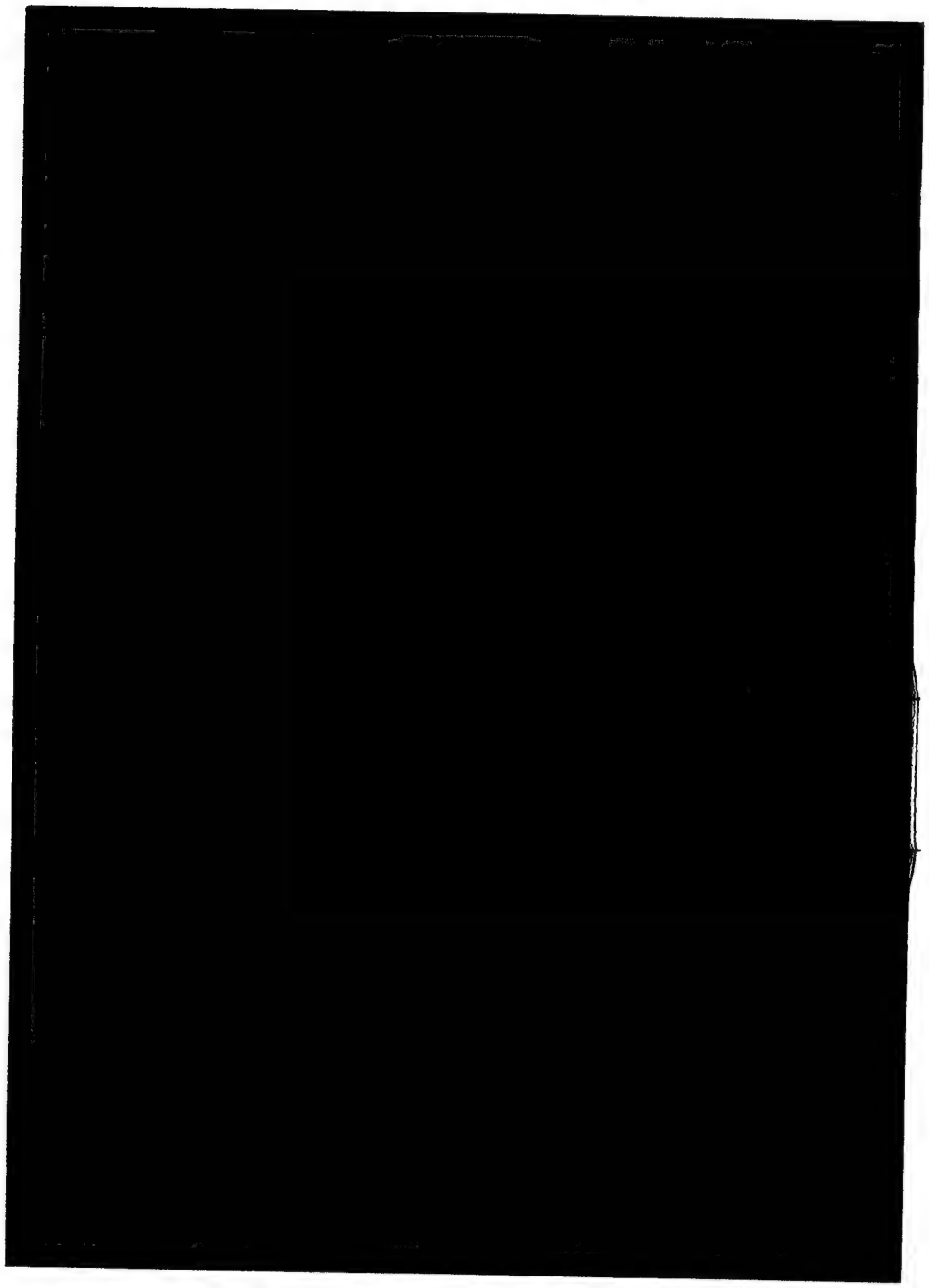

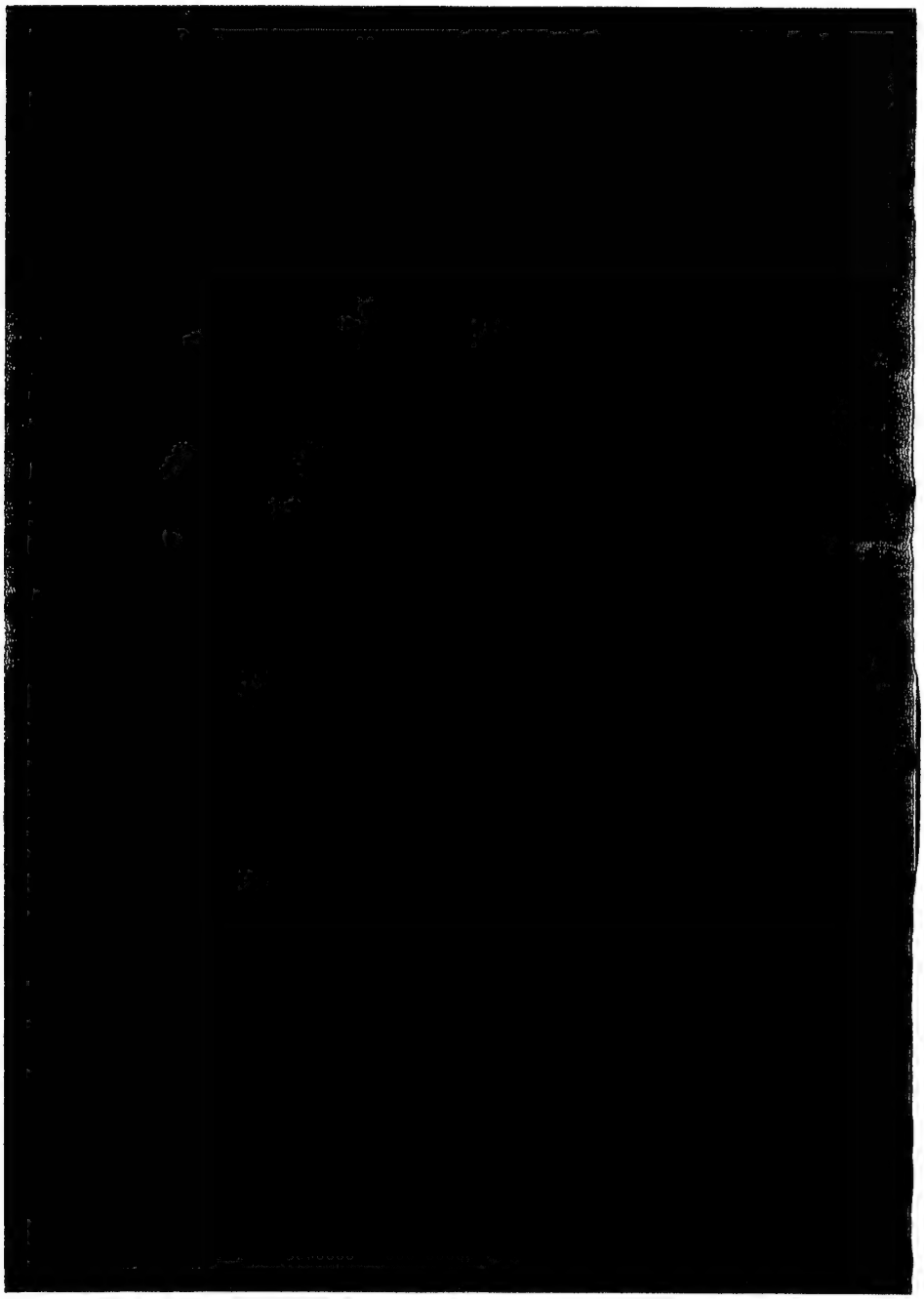

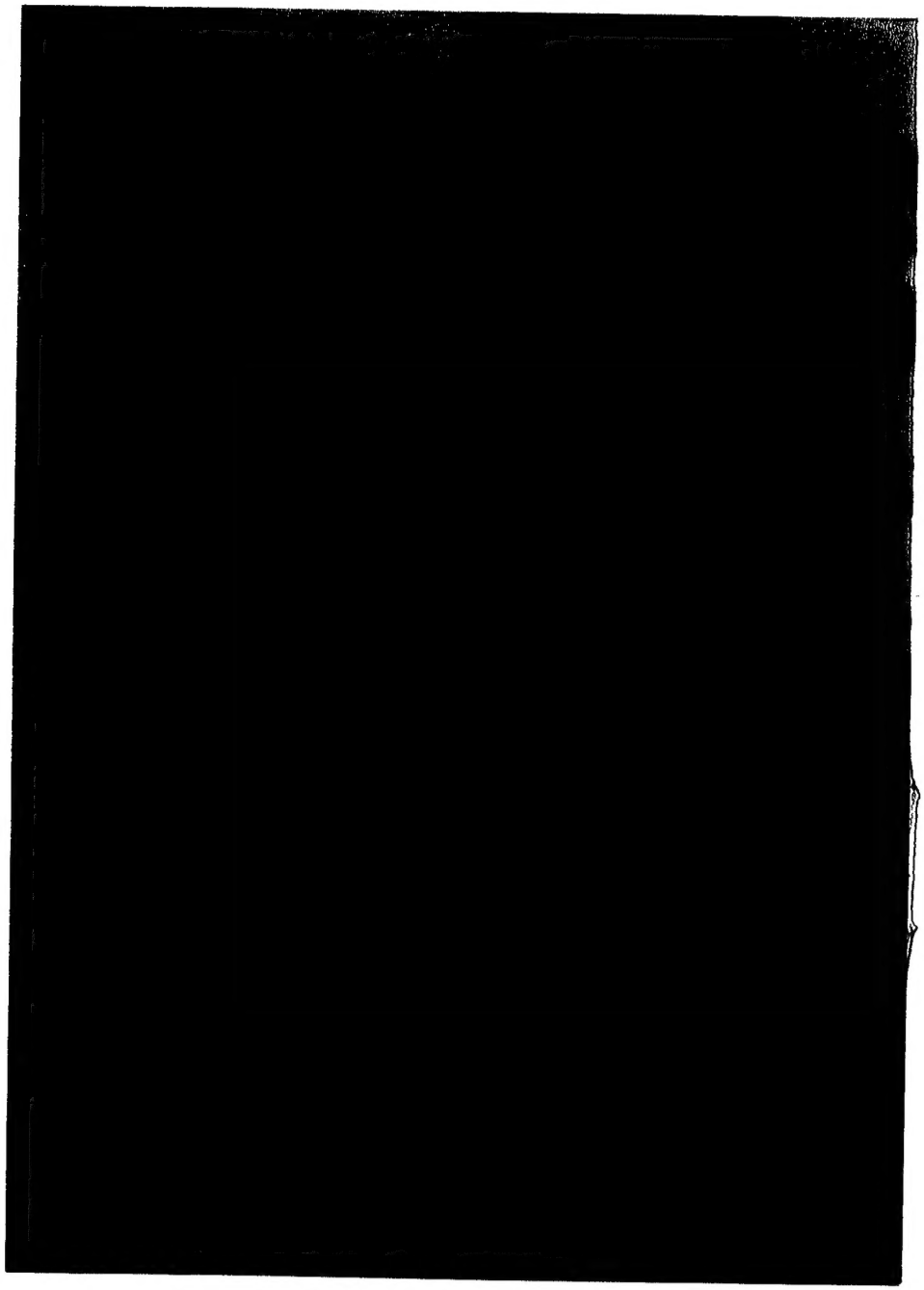

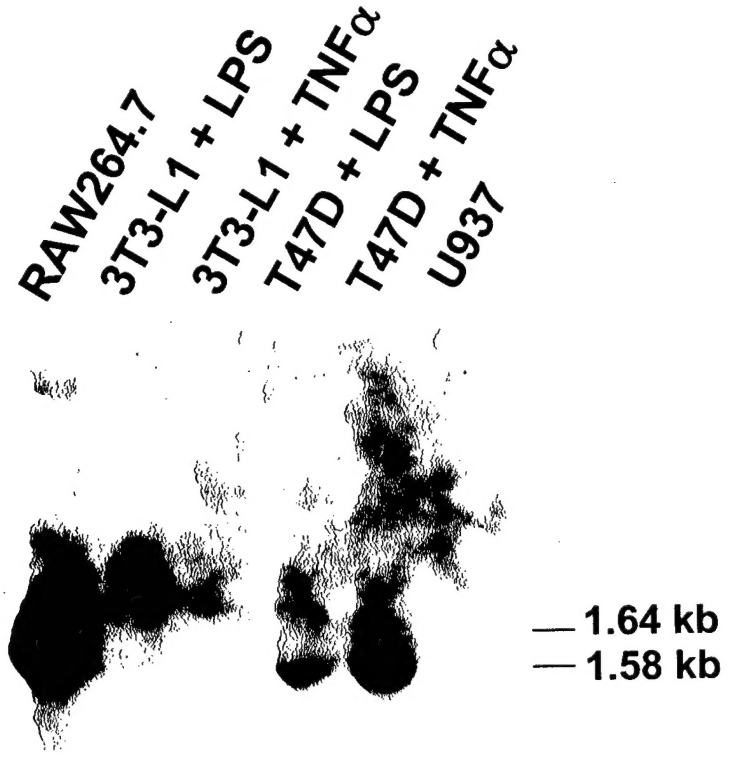
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